Association of Cell Adhesion Kinase β (CAKβ/PYK2) with Cytoskeleton

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ABSTRACT

Cell adhesion kinase β (CAKβ/PYK2), which we identified by cDNA cloning, is the second protein-tyrosine kinase of the focal adhesion kinase subfamily and has large N- and C-terminal domains in addition to the central kinase domain. In this paper, we report that CAKβ is present in association with both microtubules and microfilaments. The intracellular localization of CAKβ was studied in epithelial cells (A-431, Caco-2, and MDCK) and in nonepithelial cells (PC-12h and WFB). The immunocytochemical staining of epithelial cells revealed that CAKβ was mainly found at the cell-to-cell borders and at the perinuclear regions, although focal adhesions were also stained to some extent. The cell-to-cell interfaces were immunostained in fine dotted lines and a significant portion of CAKβ was found in association with the microtubules. Moreover, an association of CAKβ with microfilaments was found in the cytoplams. Among WFB cells only a limited number were positive for the CAKβ immunostaining at the focal contacts, although the protein was present in the tips of lamelipodia and microspikes in many cells. Furthermore, CAKβ was concentrated at the edge of ruffling membranes. When A431 cells were stimulated with epidermal growth factor, CAKβ rapidly moved to the edges of the ruffling membranes from the sites of cell-cell contacts where the protein had been present before the stimulation. Anti-actin or anti-α-tubulin antibodies coimmunoprecipitated CAKβ from a cytoskeleton-rich fraction of WFB cells. Moreover, the amount of CAKβ increased when the immunoprecipitation was carried out with the fraction prepared from the cells after stabilizing the tubular structure of the cytoskeleton. Immunoelectron microscopy showed that CAKβ was present along the microfilaments. In the WFB cells transfected with recombinant adenovirus expressing CAKβ, the protein was found at the focal adhesions of many cells and at the perinuclear region of all cells. Thus, CAKβ may associate with both microfilaments and microtubules, and play important roles for cellular movements such as membrane ruffligs, cellular attachments, and cytokinesis.

Key words : CAKβ, Microtubule, Microfilament, Protein-tyrosine kinase, Cell adhesion

INTRODUCTION

The activation of protein-tyrosine kinases (PTKs) is one of the most common signal transduction mechanisms directly coupled to receptor activation by external signals. The PTKs that do not span the plasma membrane (the so-called nonreceptor PTKs) have been classified into different subclasses (subfamilies) (for review see Hanks and Quinn[1]). CAKβ[2] (also known as PYK2[3], RAFTK[4],

and CADTK[5]) is a nonreceptor protein-tyrosine kinase of the focal adhesion kinase (FAK) family. The cDNAs of the protein have been cloned from rat[2,5], mouse[4], and human[3,4,6,7]. The amino acid sequence of human CAKβ is 95.4% identical with that of the rat CAKβ[6]. The human gene of CAKβ was mapped to chromosome 8 at p21.1[7,8] and the mouse CAKβ gene was mapped to chromosome 14[4].

CAKβ and FAK are closely related in their overall structures. Both proteins have ligand sequences for Src homology 2 and 3 (SH-2 and SH-3) domains but do not have SH-2 and SH-3 domains themselves. The two proteins have a homology over their entire length except for the extreme N-terminal portions and 10 C-terminal residues. The 88 N-terminal residues of CAKβ are entirely different from the corresponding 81 N-terminal residues of FAK[2]. CAKβ mRNA is less evenly expressed in a variety of rat organs than FAK mRNA[2]. Axons of the central nervous system, ciliated epithelial cells of the epididymis and bronchus, and microvilli of intestinal and urinary tubular epithelia are rich in CAKβ[9]. The expression of FAK is also high in the brain and was found in axons, dendrites and the cytoskeleton of astrocytes and neurons[10]. In fibroblasts, FAK is concentrated in sites of focal adhesion and participates in signal transduction through integrin-mediated cell adhesion. CAKβ/PYK2/RAFTK is highly expressed in hematopoietic cells and in megakaryocytes and B lymphocytes, the protein is coexpressed with FAK[4]. Although we showed that the tyrosine-phosphorylation of CAKβ is not enhanced by the adhesion of rat fibroblast 3Y1 cells to fibronectin-coated dishes under conditions where the tyrosine-phosphorylation of FAK is markedly stimulated[2], tyrosine phosphorylation of endogenus RAFTK was observed upon fibronectin-induced activation of human megakaryocytic cells[11]. Moreover, immunocytochemistry showed localization in focal adhesion-like structures of most RAFTK expressed in COS cells by RAFTK cDNA transfection and of RAFTK present in megakaryocytic cells. However, we found that CAKβ expressed in COS-7 cells from transfected CAKβ cDNA is present at cell-to-cell interfaces but not at focal adhesions[2]. These contradictory results prompted us to study the intracellular location of CAKβ in various cells by using several antibodies against different portions of CAKβ.

We recently reported the *in vivo* localization of CAKβ at the cytoskeleton-rich structures of cells such as axons, cilia and microvilli. This result suggests the association of CAKβ with microtubules and microfilaments. Astier et al.[12] showed that prior treatment of cells with cytochalasin B decreased the level of RAFTK phosphorylation observed on stimulating integrin and B cell antigen receptor, indicating that this phosphorylation was partially cytoskeleton-dependent.

CAKβ is activated by osmotic stress and in response to stimulation of those G-protein-coupled receptors and receptor tyrosine-kinases that are linked to phospholipase C activation and the Rho GDP/GTP exchanger[13]. It was also shown that CAKβ/PYK2 is activated by stimulation of the T cell receptor[13,15], interleukin-2 receptor[16], Fcε receptor I[17] and interferon γ-receptor[18]. CAKβ has binding sites for p130^{Cas} (Cas), Graf[19,20], Hic-5[21], and paxillin and these proteins are tyrosine-phosphorylated upon CAKβ activation[12,21-24]. Hic-5 is a CAKβ-binding protein localized at focal contacts in adherent cells[21].

In the present paper, using immunocytochemistry and immunoelectron microscopy we examined the intracellular location of CAKβ in some cell lines and in a fibroblastic cell line transfected with CAKβ cDNA. The association of CAKβ with both tubulin and actin was also studied by immunoprecipitation with an anti-CAKβ antibody. The results reported in this paper indicate that CAKβ is colocalized with microtubules and microfilaments in these cells and that CAKβ is coimmunoprecipitated with filamentous tubulin and actin. These results suggest that CAKβ participates in a signaling

pathway in close association with cytoskeletal structures.

MATERIALS AND METHODS

Cell Culture

Cell lines, A-431 (CRL 1555), Caco-2 (HTB 37), and MDCK (CCL 34), were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). A subline of PC-12h cells was provided by Dr. H. Hatanaka[25] (Osaka University, Suita, Japan). A rat fibroblast line, WFB[26] was obtained from the establisher of the line, Dr. N. Sato (Sapporo Medical University, Sapporo, Japan). A-431, MDCK and WFB cells were cultured in Dulbecco's modified Eagles' medium (DMEM) (Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated (56℃ for 30 min) fetal bovine serum (FBS) (HyClone Lab. Inc., Logan, UT, U.S.A.), penicillin and streptomycin. Caco-2 cells were cultured in DMEM with 20% FBS, and PC-12h cells were grown in DMEM with 5% horse serum (GIBCO BRL Products, Gaithersburg, MD, U.S.A.) and 5% FBS (GIBCO BRL Products). For the purpose of observing neural protrusions, PC-12h cells were cultured in the presence of nerve growth factor (NGF; Takara Shuzo, Osaka, Japan) at 10 ng/ml. Some dishes of A-431 cells were treated with epidermal growth factor (EGF; Collaborative Biomedical Products, Bedford, MA, U.S.A.) at 100 ng/ml in Ca^{2+}-, Mg^{2+}-free, phosphate-buffered saline (PBS).

Antibodies

The anti-CAKβ antibody mainly used in this study, anti-CAKβ(C-a), was raised in rabbits against a glutathione S-transferase (GST) fusion protein of residues 670-716 of rat CAKβ and was affinity-purified on a column of the immunogen covalently bound to cyanogen bromide-activated Sepharose 4B. The second anti-CAKβ rabbit antibody, anti-CAKβ(C-b), was raised against a TrpE fusion protein of rat CAKβ residues 779-1008 and was affinity-purified on a column of a GST fusion protein of the rat CAKβ C-domain as described previously[2]. The third anti-CAKβ antibody, anti-CAKβ(N), was raised against a GST fusion protein of rat CAKβ residues from minus 5 to 416; this antiserum was used without further purification. All these anti-CAKβ antibodies immunoprecipitated CAKβ of rat, mouse, human, canine, and bovine origin and were good for use in immunoblotting.

Mouse monoclonal anti-α- and β-tubulin antibodies were obtained from Amersham International plc (Buckinghamshire, UK) and Sigma Chemical Co. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit immunoglobulins and rhodamine-conjugated anti-mouse and rabbit immunoglobulins were purchased from DAKO (Copenhagen, Denmark). Monoclonal anti-FAK antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal anti-rabbit immunoglobulin (clone RG-16) conjugated with alkaline phosphatase, goat anti-mouse IgG (Fc-specific) conjugated with alkaline phosphatase, monoclonal anti-β-actin (clone AC 74), monoclonal anti-vinculin (clone hVIN-1), goat anti-mouse IgG-agarose, rabbit anti-α-catenin, and rhodamine-conjugated phalloidin were purchased from Sigma Chemical Co. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.).

Immunofluorescence microscopy and confocal laser scanning microscopy

Cells grown on glass coverslips coated with rat tail collagen[27] were fixed with cold absolute ethanol and kept at -20℃ until use. After being rinsed with PBS, the cells were incubated with Block Ace (Dainippon Pharmaceutical Co., Tokyo, Japan) at RT for 30 min. Then a primary antibody was applied. The incubation with anti-CAKβ antibodies was done overnight at 4℃ and the incubation with other antibodies was done for one hour at RT. FITC or rhodamine-conjugated antibodies were then applied for 30 min at RT. The cells were thor-

oughly washed in PBS and incubated with the secondary antibody. After being rinsed with PBS, the coverslips were mounted in 90% glycerol containing 1 mg/ml p-phenylenediamine (Kanto Chemical Co., Tokyo, Japan)/10% PBS. For double immunostaining, the following combinations of primary antibodies or stains were used; an anti-CAKβ antibody and rhodamine-phalloidin, anti-CAKβ and anti-β-actin antibodies, and anti-CAKβ and anti-β-tubulin antibodies. The samples were examined with an Olympus epifluorescence photomicroscope (Olympus, Tokyo, Japan). Immunofluorescence of some samples was imaged with a confocal laser scanning microscope (Model-2010, Molecular Dynamics, Inc., Sunnyvale, CA, U.S.A.)

Immunoprecipitation of CAKβ

Confluent monolayer cultures of cells in 9-cm dishes were washed twice with PBS and then lysed on ice in 0.5 ml per dish of a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1% Trasylol, 20 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na_3VO_4, 20 mM $Na_4P_2O_4$]. A 2.5% rat brain lysate was prepared in the lysis buffer by the use of a Teflon pestle in a glass homogenizer. The lysates were subjected to centrifugation at 15,000 x *g* for 20 min at 4°C to obtain clarified lysates. CAKβ was immunoprecipitated by mixing anti-CAKβ bound to protein A-Sepharose with either 1 mg or 2.5 mg of protein of the clarified lysates and by incubating for 2 h at 4°C on a rotating platform. The anti-CAKβ beads were prepared for each assay by mixing either 2 μg of protein of affinity-purified anti-CAKβ or 4 μl of anti-CAKβ serum with 10 μl (packed volume) of protein A-Sepharose and washing the Sepharose beads with the lysis buffer. As a control, preimmune serum beads were prepared for each assay by mixing 4 μl of preimmune serum with 10 μl of protein A-Sepharose. Immunoprecipitates were washed three times with the lysis buffer, and proteins were separated by SDS-PAGE according to the method of Laemmli and Favre[28]. The separated proteins were blotted onto PVDF membranes (Immobilon-P, Millipore, Bedford, U.S.A.). The membranes were blocked with 3% bovine serum albumin (BSA) in TBST [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] for 20 min at 60°C and then probed with a primary antibody in TBST containing 1% BSA for 1 h at RT. For the immunoblotting, affinity-purified anti-CAKβ(C-a) antibodies were used at 1 μg of protein per ml and anti-CAKβ serum was used at 200-fold dilution. The membranes were washed with TBST three times and probed again with a second antibody conjugated with alkaline phosphatase in TBST for 1 h, followed by washing three times in TBST. Positive bands were detected by incubation in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Immunofluorescence and immunoelectron microscopy for CAKβ in Caco-2 cells treated with cytoskeleton-stabilizing buffer

Caco-2 cells were cultured in a 35-mm dishes with or without a glass coverslip for immunofluorescence and immunoelectron microscopy, respectively. The cells were treated with a cytoskeleton-stabilizing buffer[29] [CS buffer; 0.15 M NaCl, 1mM $MgCl_2$, 1mM EGTA, 20 mM HEPES, 2 mM ATP, 1 M glycerol, 0.5% Triton X-100, 0.1 mM PMSF (pH 6.9)] for 15 min at RT. The cells were then fixed with a solution [0.15 M NaCl, 1mM $MgCl_2$, 1mM EGTA, 20 mM HEPES, 1 M glycerol, 1% paraformaldehyde, 0.125% glutaraldehyde (pH 6.9)] for 15 minutes at RT and rinsed with PBS. Block Ace was applied to the cells for 1 h at RT. Thereafter, the cells were incubated with an anti-CAKβ (C-a) (40 μg/ml) overnight at 4°C, after which they were rinsed several times with PBS. Anti-rabbit IgG antibody conjugated with 10 nm colloidal gold (British BioCell, Cardiff, UK) was applied overnight at 4°C. After rinsing the cells with PBS, they were

postfixed in 2% osmium tetraoxide in the buffer, dehydrated and embedded *in situ* in Epon 812. For immunofluorescence microscopy, FITC-conjugated anti-rabbit Ig was applied for 30 min at RT. In order to examine the colocalization of CAKβ and actin or tubulin, double immunostaining was conducted The cells were incubated with rhodamine-phalloidin or anti-β-tubulin antibodies for 30 min at RT. Rhodamine-conjugated anti-mouse Ig was then applied for 30 minutes at RT. After being rinsed with PBS, the coverslips were mounted in 90% glycerol containing 1 mg/ml p-phenylenediamine. The samples were imaged with a Zeiss confocal laser scanning microscope 410 (Zeiss, Germany).

CAKβ-expressing recombinant adenovirus

A *Bst*UI-*Dra*III fragment of rat CAKβ cDNA, containing the entire coding sequence, was blunted and inserted into the *Swa*I site of the adenovirus cosmid cassette, pAxCAwt. Recombinant adenovirus for expression of CAKβ, designated AxCASCAKβ, was obtained by transfecting 293 cells with the above cosmid together with adenoviral DNA cut with *Eco*T22I, according to the COS-TPC method. Recombinant adenovirus was used after cloning of the virus by limited dilution. For infection with the adenovirus to WFB cells, the medium was taken away from monolayer cells growing at 70% confluence either in 10 cm dishes or on glass coverslips placed in 35 mm dishes. Then, cell infection with the recombinant adenovirus was done in 50 μl and 0.5 ml of culture medium for the cells on a coverslip and the cells in a 10 cm dish, respectively, at a multiplicity of infection of about 10. One h after the infection, culture medium was added to the cells.

RESULTS

Expression of CAKβ in cell lines

Although we previously showed the expression of the CAKβ mRNA in several cell lines, including fibroblasts and lymphocytes[2], epithelial cell lines were not extensively examined for CAKβ expression. In the COS-7 cells transfected with the CAKβ cDNA, the expressed CAKβ was found localized in the cell membrane at cell-to-cell contacts. This finding was one of the reasons why we named the protein cell adhesion kinase β (CAKβ). The presence of CAKβ at cell-to-cell contacts prompted us to explore the subcellular localization in other epithelial cell lines (Caco-2, MDCK, and A 431), a neuronal cell line (PC12) and a fibroblastic cell line (WFB). Figure 1 compares the amounts of CAKβ expressed in these cell

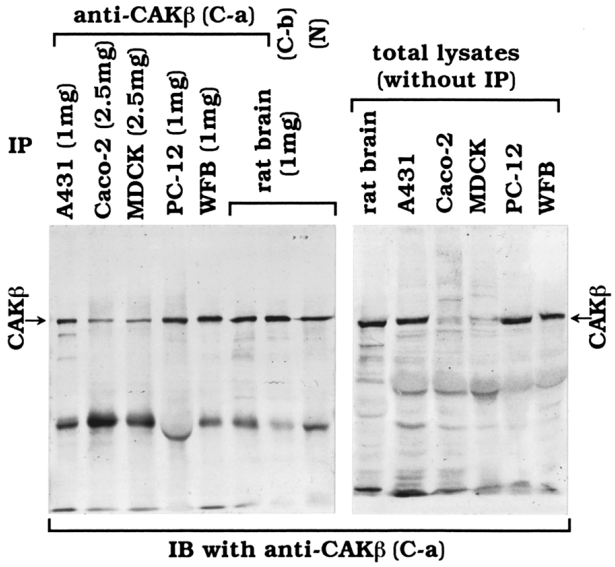

Fig. 1 Expression of CAKβ in various cultured cell lines and rat brain. The indicated amounts of the lysates prepared from the indicated cells or rat brain were subjected to immunoprecipitation with the indicated anti-CAKβ antibodies as described in MATERIALS AND METHODS. The immunoprecipitates (left panel) and 16.5 μg protein of the total lysates (right panel) were subjected to SDS-PAGE in 7.5% gel, blotted and probed with anti-CAKβ(C-a). The position of CAKβ is indicated by arrows.

lines. Caco-2 and MDCK cells contained smaller amounts of CAKβ as can be seen by comparing the CAKβ bands of these cells, which were made visible by immunoblotting after separation of total cellular proteins, with the CAKβ bands of A-431, PC-12h, and WFB cells (Fig. 1, right); this is also evident in the immunoblot of the anti-CAKβ immunoprecipitates

separated by SDS-PAGE (Fig. 1, left). More than two times of the amounts of the cell lysates from Caco-2 and MDCK cells were necessary to detect the CAKβ bands by immunoblot after immunoprecipitation with anti-CAKβ and separation by SDS-PAGE as compared with the amounts of the lysates from A-431, PC-12h, and WFB cells. The three rabbit anti-CAKβ antibodies, (C-a), (C-b), and (N), directed to different portions of CAKβ precipitated CAKβ equally well from the rat brain lysate (Fig. 1); however, only the antiserum raised against the CAKβ(N) could efficiently immunoprecipitate CAKβ without affinity purification.

Subcellular localization of CAKβ in cell lines

CAKβ in various cell lines was immunostained to study its subcellular localization. First, we looked at the staining of Caco-2 cells in order to examine the specificity of the anti-CAKβ antibodies in immunocytochemical staining. Caco-2 cells were selected because among the cell lines examined the positive staining of cell-to-cell contact (arrows in Fig. 2) observed in the CAKβ cDNA transfected COS7 cells was most clearly observed in Caco-2 cells. As shown in Fig. 2A, intense staining by the anti-CAKβ(C-a) antibody was observed not only at the sites of cell-to-cell contact but also at the circumference of the nucleus. In addition, many thin lines running through the cytoplasm were stained by the anti-CAKβ antibody. No positive staining was found in Caco-2 cells when the primary antibody was omitted (Fig. 2B) or when the anti-CAKβ antibody was pretreated with a 10-fold molar excess of the antigen, GST-CAKβ (670-716) (Fig. 2C). As shown in Fig. 2D, the anti-CAKβ(C-b) antibody also intensely immunostained the sites of cell-to-cell contact of Caco-2 cells. This antibody against the C-terminal portion of CAKβ stained the nucleus in addition to the perinuclear region but no positive line in the cytoplasm was found. The immunostaining of Caco-2 cells with anti-CAKβ(N) antibody was almost the same as that with the anti-CAKβ(C-a); the nuclear circumference and the sites of cell-to-cell contact were stained (Fig. 2E). The nucleus was stained with anti-CAKβ(C-b) but not with anti-CAKβ(C-a) or (N). In the experiments shown below we used anti-CAKβ(C-a) in the immunocytochemical staining of CAKβ. In MDCK cells, dotted lines of immunostaining were observed at the sites of cell-to-cell contact (Fig. 2F, arrows). In A-431 cells, immunoreactive dots were also observed at the sites of cell-to-cell contact although the number of dots was fewer and each dot was coarser than those found in Caco-2 and MDCK cells (Fig. 2G). On the other hand, in PC-12h cells the cytoplasm and the neural protrusions were strongly positive in the immunostaining (Fig. 2I).

In fibroblasts, it is known that focal adhesions develop well in the bottom of the cells and that vinculin, paxillin, and FAK are concentrated at sites of focal contacts[30]. As CAKβ and FAK are closely related in their overall structures, we examined the localization of CAKβ in the focal adhesion. In WFB cells, as shown in Fig. 3, filamentous networks of immunoreactive materials, CAKβ, were found in their cytoplasms, especially in the perinuclear region. Fig. 3B shows that most CAKβ was not present in the focal adhesions, which were made visible by the positive stainings of paxillin. Other antibodies such as CAKβ(C-b) and (N) did not stain focal adhesions either (data not shown). However, as shown in Fig. 3C and 3E, some CAKβ was concentrated at the leading edges of the cells and at the ruffling membranes, where proteins at focal adhesions such as paxillin and vinculin are known to be concentrated.

Translocation of CAKβ to the ruffling membranes in A431 cells

As positive staining for CAKβ in Caco-2 cells was found in the ruffling membranes, we examined the translocation of the protein to the ruffling in A431 cells stimulated with EGF. A large portion of CAKβ in A431 cells was pres-

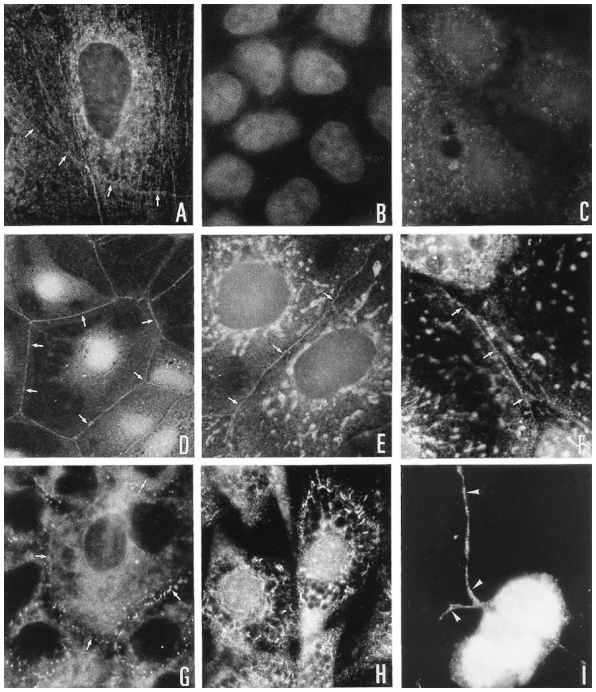

Fig. 2 Subcellular localization of CAKβ in various cells shown by immunostaining. Immunostaining of Caco-2 cells using purified anti-CAKβ(C-a) antibody (A): dotted lines are observed in the cytoplasm; the nuclear circumference is strongly stained; arrows show cell-to-cell contact. Immunostaining of Caco-2 cells without primary antibody (B): no specific staining is observed. No positive staining of Caco-2 cells is observed when anti-CAKβ(C-a) is mixed with a10-fold molar excess of the immunogen prior to the staining (C). Immunostaining of Caco-2 cells using anti-CAKβ(C-b) (D): this antibody positively stains the cell-to-cell contact (arrows) and perinuclear regions; nuclei are nonspecifically stained. Immunostaining of Caco-2 cells using anti-CAKβ(N) (E): staining with this antibody gave a result similar to that with anti-CAKβ(C-a); arrows show cell-to-cell contacts. Anti-CAKβ(C-a) is used for immunostaining of MDCK (F), A431 (G), WFB (H), and PC12h cells (I): arrows indicate the sites of cell-to-cell contact positively stained with the antibody; arrowheads in I point to the positively stained, dotted lines at neural protrusions. Magnification of all figures is x1031.

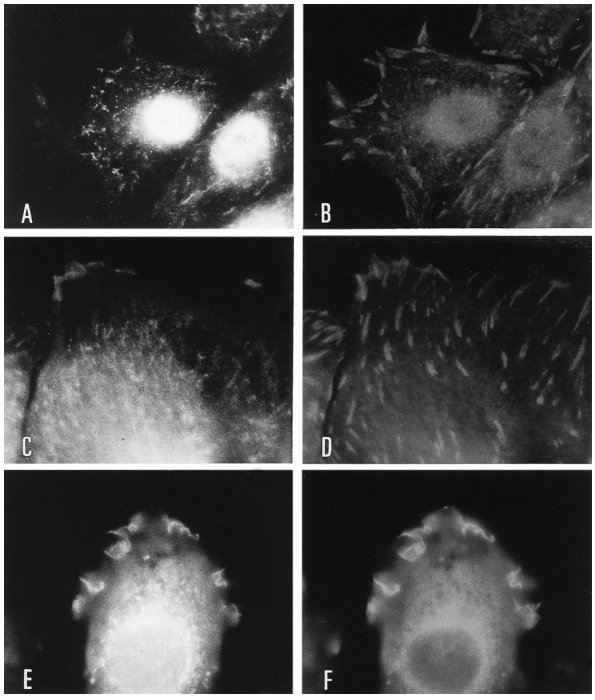

Fig. 3 Immunolocalization of endogenous CAKβ at the leading edge and ruffling membrane in WFB fibroblasts. WFB cells were doubly immunostained with anti-CAKβ(C-a) (A) and anti-CAKβ (N) (C and E) and either anti-paxillin (B and D) or anti-vinculin (F). The stained cells were viewed under a fluorescence microscope. Magnification of all figures is x312. CAKβ was not found at focal adhesions (A and C), but was found at the leading edge (C) and ruffling membrane (E).

ent at the cortical region of the cells and the other portions of CAKβ were distributed evenly in the cytoplasm (Fig. 4A). Filamentous actin was also intensely stained at the cortical region of A-431 cells (Fig. 4B). When A431 cells were stimulated with EGF in Ca^{2+} and Mg^{2+}- free PBS, the cells were rapidly freed from cell contacts. Confocal laser scanning microscopy revealed that CAKβ was concentrated at the edges of the ruffling membranes 4 minutes after EGF addition (Fig. 4C) and that filamentous actin also concentrated at the same site as CAKβ (Fig. 4D). Ten minutes after EGF addition, the cells became spherical and the ruffling membranes disappeared (data not shown). During this dramatic morphological change of the cells, the level of CAKβ tyrosine-phosphorylation was not enhanced by the EGF stimulation (data not shown).

CAKβ colocalized in the microfilaments and microtubules in epithelial cells

The results described above and our results reported previously revealed localization of CAKβ *in vivo* at the cytoskeleton-rich struc-

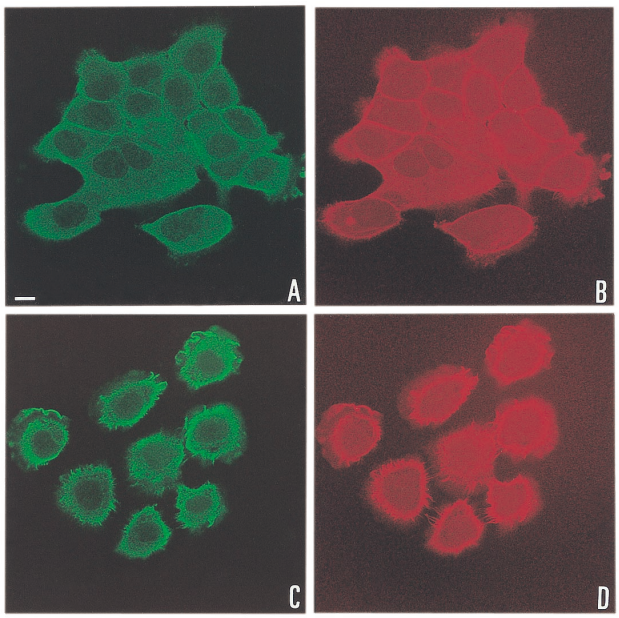

Fig. 4 Co-localization of CAKβ and microfilaments in A431 cells stimulated with EGF. Doubly stained confocal laser scanning micrographs with anti-CAKβ (green; A and C) and with rhodamine-phallodin (red; B and D) are shown. Cells before (A and B) and 4 minutes after (C and D) the treatment with 100 ng/ml EGF were stained. Both CAKβ and microfilaments were concentrated at the edges of ruffling membranes in the EGF-stimulated cells. Bar represents 10 μm.

tures of cells such as axons, cilia and microvilli[9]. These results suggested an association of CAKβ with actin and/or tubulin. Therefore, by double immunostainings, we examined the possible colocalization of CAKβ and actin and of CAKβ and β-tubulin in Caco-2 cells by the use of confocal laser-scanning microscopy. As shown in Fig. 5A, CAKβ was fiberously stained at the cell cortex and at the perinuclear regions. Actin filaments stained with rhodamine -phalloidin were observed mainly underneath the cell membrane as thick bundles of stress fibers (Fig. 5B). It is well known that actin filaments are rich at the adherence junctions and underneath the cell membrane. The colocalization of CAKβ and actin was observed at the cell cortex and the cell membrane as well as at stress fibers (Fig. 5C). At the perinuclear region, actin was not well developed and linear green stainings were found there (Fig. 5C). Although the nuclei seemed to be positively stained with anti-CAKβ, we interpreted this nuclear staining to be nonspecific because the nuclei were also nonspecifically stained, as shown in Fig. 5B (actin) and 5D (normal rabbit serum).

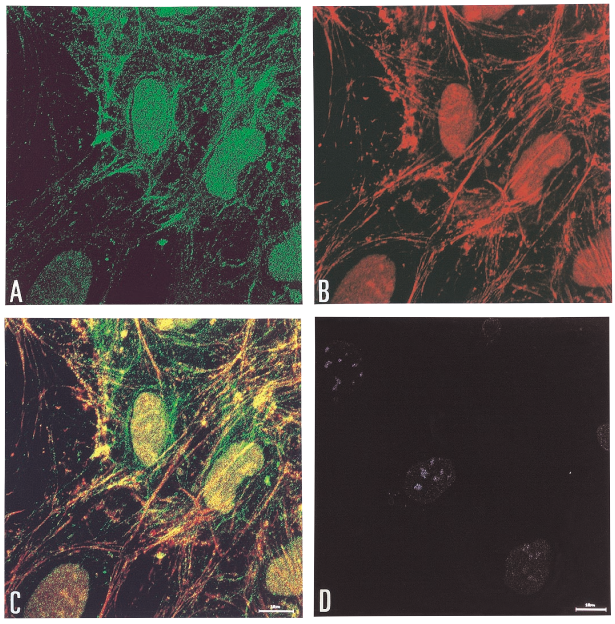

Fig. 5 Co-localization of CAKβ and microfilaments in Caco-2 cells shown by staining with anti-CAKβ and phalloidin. Cells were treated with cytoskeleton-stabilizing buffer. Doubly stained confocal laser scanning micrographs with anti-CAKβ (green; A), rhodamine-phalloidin (red; B), and both anti-CAKβ and rhodamine-phalloidin (C) are shown. Normal rabbit serum was used as a primary antibody in the control (D). Colocalization of the green FITC and the red rhodamine signals produced a yellow signal. Bars represent 10 μm

The association of CAKβ with microfilaments at the cell cortex of Caco-2 cells was also shown by immunoelectron microscopy. As shown in Fig.6, gold particles (10 nm) were present along thin filaments, which seemed to be microfilaments. Characteristically, clusters of gold particles were present on the short filaments branching from the long microfilaments, as indicated by arrows in Fig. 6.

In our study on the tissue distribution of CAKβ[9], it was found that CAKβ is mostly present in association with nerve axons and cilia. These results indicated the possibility

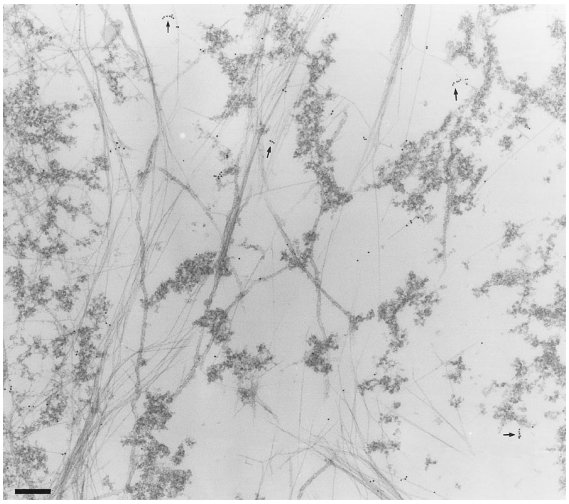

Fig. 6 Immunoelectron micrograph of CAKβ in Caco-2 cells. The cells were treated with cytoskeleton -stabilizing buffer before fixing. 10-nm gold particles lined up along branched microfilaments (arrows). Bar represents 200 nm.

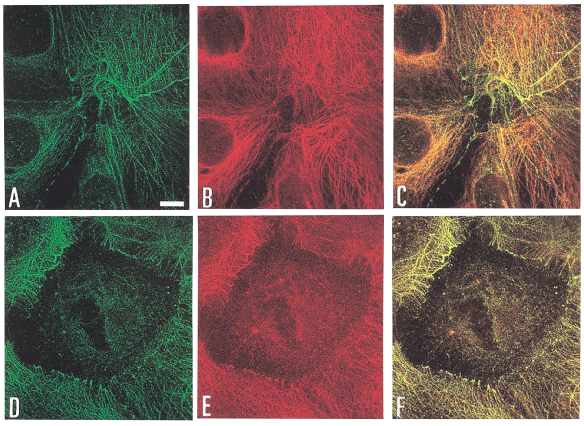

Fig. 7 Co-localization of CAKβ and β-tubulin in Caco-2 cells shown by immunostaining. Doubly immunostained confocal laser scanning micrographs of CAKβ (green; A and D), β-tubulin (red; B and E), and both CAKβ and β-tubulin (C and F) are shown. Colocalization of the green FITC and the red rhodamine signals produced a yellow signal. Bars represent 10 μm (A–F).

that CAKβ and microtubules have some structural and functional associations. Therefore, we examined the colocalization of CAKβ and tubulins in Caco-2 cells. Confocal laser scanning microscopy revealed that CAKβ aligned toward cell membranes as dotted lines and that every line pointing toward the cell membrane seemed to face the immunoreactive staining at the sites of cell-to-cell contact (Fig. 7A). Although the network stained in the cytoplasm with anti-α-tubulin (Fig. 7B) was denser than that made visible with anti-CAKβ, colocalization of both proteins was observed as thick lines in the cytoplasm, as shown in Fig. 7C by yellow lines. Figures 7D, E, F show CAKβ in the cells surrounding the dividing cells of the prometaphase. CAKβ in these cells was concentrated at the cell periphery, seemingly pulling and supporting the dividing cells. This CAKβ in the surrounding cells had more exact coincidence in its location with α-tubulin compared with the CAKβ observed in Fig. 7A (Fig. 7F).

Overexpressed CAKβ from infected recombinant adenovirus localized in focal adhesions

WFB cells infected with CAKβ-expressing recombinant adenovirus were studied by immunocytochemistry. As described above, endogenous CAKβ in WFB cells was rich in perinuclear regions and in the tips of microspikes and lamellipodia. However, CAKβ overexpressed in WFB cells was found at focal contacts by immunostaining (Fig. 8C and 8E), where FAK was also found (Fig. 8D and 8F). Vinculin and paxillin also colocalized in the focal contacts where CAKβ was located (data not shown). After the cells had been fixed and lysed by the use of nonionic detergent, cytoplasmic organella and nuclei disappeared but the structures binding tightly to extracellular matrix, such as focal contacts, remained. In those ghost cells, CAKβ was clearly observed at the sites of focal contacts located by anti-FAK antibody (Fig. 8E and 8F). In the mock transfected cells, only limited CAKβ was found at the focal contacts (Fig. 8A and 8B). Although CAKβ was found at the focal contacts of most WFB cells transfected with CAKβ cDNA, only a small fraction of 3Y1 cells (mouse fibroblastic cells) infected with the same CAKβ-expressing recombinant adenovirus possessed the CAKβ-positive focal contacts (data not shown).

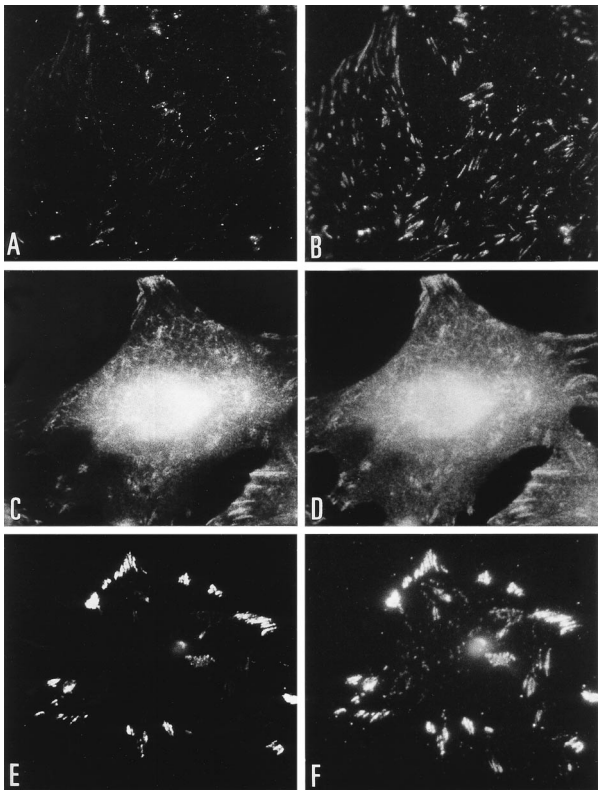

Fig. 8 Localization in focal adhesions of CAKβ overexpressed by the infection of CAKβ-expressing recombinant adenovirus. WFB cells were infected with either recombinant adenovirus expressing CAKβ (C, D, E and F) or control vector adenovirus (A and B). The cells were cultured, fixed with ethanol at -20 °C 24 h after the infection, and doubly immunostained with anti-CAKβ(N) (A, C and E) and anti-FAK (B, D and F). Doubly immunostained cells are shown in A and B, C and D, and E and F. The ethanol-fixed cells shown in E and F were immunostained after lysis with nonionic detergent to wash away cytoplasmic organellae and nuclei. Magnification of figures A and B is x165 and that of figures C-F is x412.

DISCUSSION

In epithelial cells such as Caco-2, A431, and MDCK, we found endogenous CAKβ to be mainly localized at the sites of cell-to-cell contacts and in the cell cortex. In the fibroblastic cells, we also found that a large portion of CAKβ was present at the perinuclear region and in the cell cortex. We used three polyclonal antibodies against different portions of CAKβ. With all three antibodies, immunostaining was positive at the sites of cell-to-cell contacts in epithelial cells and at the cell cortex in fibroblastic cells. It is of interest that leading edges of cells like ruffling membrane and microspikes were strongly immunostained with all three antibodies and in all the cell lines examined. These structures are well known as structures containing dense meshworks of actin filaments. Therefore, an association of CAKβ with actin filaments was suspected. In fact, we showed in this paper that CAKβ colocalized with actin filaments in Caco-2 cells and that CAKβ translocated to the ruffling membrane in A431 cells soon after cell stimulation with EGF. Moreover, immunoelectron micrograph showed the presence of gold particles along the branched microfilaments. An immunoprecipitation of actin filaments coprecipitated a portion of CAKβ. It was reported by Astier et al.[12] that the RAFTK phosphorylation in megakaryocytes induced by either integrin- or B cell receptor-stimulation was decreased by pretreating the cells with cytochalasin B. These results were interpreted by the authors to suggest that the tyrosine phosphorylation of RAFTK required the formation of a RAFTK-cytoskeleton complex. All these findings are compatible with our immunohistochemical findings that in rat tissues[9] CAKβ is present in cells in association with the brush border of absorptive epithelial cells in small intestine and also with the brush border of proximal tubular cells in kidney, in which microvilli develop well. Microvilli are well known to consist of microfilaments. Although we did not present the data, it was not possible with any one of anti-CAKβ antibodies to immunoprecipitate all CAKβ present in the cell lysates. Even after the immunoprecipitation had been repeated several times with one antibody, the cell lysates still contained a significant amount of CAKβ, which could be immunoprecipitated with other antibodies. This result suggests that different forms of CAKβ, which may be conformationally different and/or bind to other proteins, exist in the cell. Thus, some portion of CAKβ is present in the cell in association with microfilaments.

Although Li et al.[11] reported that CAKβ/RAFTK expressed in COS cells from transfected CAKβ cDNA was present at focal adhesions, we found very little amount of CAKβ at the cell bottom in transfected COS-7 cells[2], where FAK was located. In this study, we showed by the use of anti-CAKβ(N) antiserum that CAKβ was present both at focal adhesions and at perinuclear regions in WFB cells transfected with CAKβ cDNA (Fig. 8). In 3Y1 cells, a much smaller portion of CAKβ expressed from transfected cDNA was present at focal contacts (data not shown). Anti-CAKβ(N) was found to be much better than anti-CAKβ(C-a) and anti-CAKβ(C-b) when used to detect the CAKβ at focal contacts. Thus, it is possible that the reported discrepancy in the presence of CAKβ at focal contacts in COS cells[2,11] results from the antibodies used in these studies. Li et al.[11] studied the localization of tagged RAFTK and used fibronectin-coated coverslips, indicating possible differences in the experimental conditions from the ones we used. Salgia et al.[22] showed that the levels of RAFTK expression varied among cell lines; being high in megakaryocytic cells, moderate in hematopoietic cells, and low in adherent fibroblasts. They also reported an association of RAFTK with paxillin in hematopoietic cells. In megakaryocytes, it was shown that RAFTK colocalized with paxillin. As with FAK, the C terminus of RAFTK binds directly to the N terminus of paxillin. Salgia et al.[22] reported that RAFTK binds to a region present in the amino acid residues 100-227 of paxillin. Their results suggest that RAFTK binds to paxillin at a different site from the one where FAK binds; it was reported that FAK binds to paxillin at a region present in the amino acid residues 51-315[31]. These differences in the binding sites are a possible reason for the tyrosine phosphorylations of paxillin at different residues by FAK and RAFTK[22]. Recently, we showed the association of CAKβ with a paxillin-related protein, Hic-5, which was found to localize at focal adhesions[21]. This result suggests that some CAKβ(RAFTK/PYK2) should exist at focal contacts although the amount localizing there may depend on the cell line. CAKβ may play an important role in the signal transductions from focal contacts to actin filaments.

In this study, we also showed the association of CAKβ with microtubules. This finding is consistent with our earlier finding that CAKβ expression is evident in cells with microtubule-rich structures, such as the cilia of bronchial epithelia and of epididymis, and the axons of neurons[9]. The observation that the proteins participating in the intracellular signal transduction associate with the cytoskeleton is not new. Reszka et al.[32] found that about one-third of mitogen-activated protein kinase (MAPK) is present in association with microtubules. MAPK activities are known to be affected by cytoskeletal alteration. MAPK and N-terminal Jun kinase have been identified as the downstream targets of CAKβ/PYK2 activation[33].

The association of CAKβ with microtubules and microfilaments found in this study is most likely an indirect one; CAKβ may bind to some unidentified protein which associates with these cytoskeletons directly or indirectly. We showed that anti-CAKβ co-immunoprecipitated filamentous tubulins or actins. In immunoelectron microscopy, clusters of CAKβ were found at a small distance from microfilaments, suggesting an indirect association of CAKβ with microfilaments.

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